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Combined Sequential Treatment with Interferon and dsRNA Abrogates Virus Resistance to Interferon Action

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ABSTRACT

Many viruses have evolved mechanisms to resist the action of interferon (IFN). These include production of viral gene products that sequester double-stranded RNA (dsRNA) and of small helical RNA. These potentially prevent activation of dsRNA-dependent pathways of IFN action or block expression of cellular genes activated exclusively by dsRNA that may contribute to the antiviral state. Thus, dsRNA might be rate limiting in the development of an IFN-mediated antiviral state. In support of this hypothesis, dsRNA added exogenously to IFN-treated cells in the form of poly(rI):poly(rC) is shown to establish in a dose-dependent manner an antiviral state against two viruses otherwise highly refractory to IFN action, avian reovirus (ARV) and Newcaste disease virus (NDV). Cells exposed singly to high doses of IFN or dsRNA reduced the plaque-forming capacity of these viruses on chicken embryo cells 2-fold. When used in combination, there was up to a 100-fold reduction. In order to abrogate IFN resistance, dsRNA must be added after, not before, an IFN-mediated latent antiviral state is established. dsRNA added exogenously is thought to achieve the threshold required for activation of dsRNA-dependent pathways of IFN action or to induce some dsRNA-stimulated gene whose product acts synergistically with that of some IFN-stimulated gene. The combined sequential treatment with IFN and dsRNA may be useful in overcoming the anti-IFN activity of viruses of clinical interest or in other clinical conditions.

INTRODUCTION

As PART OF INNATE IMMUNITY, the interferon (IFN) system constitutes an early response defense mode against viruses.[1] Dominant among mechanisms used by the IFN-treated cell to block viral replication are latent antiviral states that are subsequently activated by viral double-stranded RNA (dsRNA) extant in the genome or produced during replication of the invading virus. These involve, at the least, the IFN-induced protein kinase PKR[2–4] and 2′,5′-oligoadenylate synthetase (2′,5′-OAS)[5] pathways, both of which result in the downregulation of protein synthesis and the resultant compromise of viral replication.[1] However, many viruses have evolved anti-IFN defense mechanisms that thwart this antiviral action, blunting an otherwise effective system and undoubtedly limiting its clinical use. One of the most successful of these mechanisms is exemplified by the almost absolute resistance to IFN action displayed by some reoviruses, in particular, avian reoviruses (ARV).[6–8] The S1133 strain of ARV[9] is highly refractory to the action of chicken IFN-α (ChIFN-α),[10] resisting up to 2000 U/ml in cultures of primary chicken embryo cells (CEC). In many experiments there was little or no loss in the efficiency of plaque formation.[8] The refractory nature of ARV to IFN action in CEC has been reported earlier,[6] and more recently using virus yield reduction assays.[7] The latter study demonstrated *in vitro* that the ARV-S1133-encoded σA core protein,[11] comparable in action to its mammalian counterpart, σ3,[12,13] binds dsRNA irreversibly and suggested that σA may antagonize the IFN-induced antiviral response against ARV by blocking the activation of PKR,[7] like its mammalian equivalent.[13] Earlier studies with 2-aminopurine, an inhibitor of PKR effective both *in vitro* and *in vivo*,[14] had led us to postulate the existence of PKR in chicken cells,[15] but the presence of an activity in IFN-treated CEC that blocked activation of PKR by dsRNA in a mouse cell extract precluded its demonstration (P.I. Marcus, M.J. Sekellick, and J. Lucas-Lenard, unpublished observations). Martínez-Costas et al.[7] have since provided the first direct evidence for an avian PKR in the form of a 70-kDa dsRNA-binding phosphoprotein found in extracts of IFN-treated CEC.

Like Martínez-Costas et al.,[7] we hypothesize that the resistance of ARV to IFN action reflects the sequestration by σA

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protein of viral dsRNA normally available for activation of latent IFN-mediated dsRNA-dependent antiviral mechanisms. Indeed, Beattie et al.[16] have shown that expression of the human reovirus *S4* gene that encodes the dsRNA-binding protein $\sigma 3$ reverses the IFN-sensitive phenotype of vaccinia virus which had been deleted of the dsRNA-binding protein encoded by its *E3L* gene.

Paradoxically, ARV is highly refractory to IFN action[6–8] despite being an excellent inducer of IFN because of its content of genomic dsRNA.[17] We infer that activation of dsRNA-dependent mechanisms of IFN action requires a higher threshold of dsRNA than the single molecule that suffices for IFN induction[18,19] and reasoned that it might be possible to achieve this higher threshold by adding dsRNA to cells exogenously. The added dsRNA might exceed the dsRNA-binding capacity of the ARV σA protein and thereby cause activation of the latent antiviral state. This report provides data to support this hypothesis and demonstrates for the first time that a viral anti-IFN action mechanism in cells can be overcome in a dose-dependent manner by adding dsRNA exogenously to IFN-treated cells.

MATERIALS AND METHODS

Cells and viruses

Primary cells from 9-day-old or 10-day-old chicken embryos were used to prepare confluent monolayers of cells for plaque-reduction assays as previously described.[20] Eggs were from specific pathogen-free flocks of Charles River SPAFAS, Inc. (Storrs, CT). Chicken embryo cells were plated at 10^7 cells in 50-mm dishes to achieve confluent monolayers and used 1 or 2 days later for the plaque-reduction assays. ARV strain S1133[9] was obtained from Louis van der Heide (University of Connecticut, Storrs) and grown and assayed as described.[17] The growth and assay of Newcastle disease virus (NDV)-Calif. has been described.[21] Avian influenza virus (AIV), strain A/TY/ONT/7732/66 (H5N9),[22] and vesicular stomatitis virus (VSV)-IN-HR[23] were propagated and assayed as cited. Neutral red was used as a vital stain to enhance plaque visibility for counting.[20]

IFN: Source and assay

Recombinant chicken IFN-α (rChIFN-α) was obtained from transfected COS cells[10] and processed as described previously.[20] Detailed procedures for plaque-reduction assays typical of those described here have been reported.[24] VSV is considered highly sensitive to the action of IFN and is used as the reference standard to calibrate the activity of rChIFN-α in plaque-reduction assays.[20]

dsRNA

Throughout this study, dsRNA was used in the form of high molecular weight poly(rI):poly(rC) (Amersham Pharmacia Biotech, Uppsala, Sweden) complexed with high molecular weight DEAE-dextran (10 μg/ml) (Amersham Pharmacia Biotech) to render the helical RNA resistant to dsRNase,[25] a necessity in cells capable of producing a potent dsRNase on exposure to dsRNA.[26] PolyICLC, a complex of poly(rI):poly(rC) with polylysine and carboxymethylcellulose, also was resistant to dsRNase.[27] It was a gift from Hilton B. Levy (NIAID, Bethesda, MD).

RESULTS

Range of virus sensitivity to the action of IFN on the same host cell

Figure 1 illustrates the broad range of responses that viruses may display to the action of IFN. The host cells, CEC, and batch of rChIFN-α were constant throughout this study, allowing a direct comparison between viruses. Some viruses, like VSV, represent the epitome of sensitivity and often are used to define a unit of IFN activity, that is, the dose of IFN that will reduce the plaque titer by 50% (PR_{50}).[20] For AIV, one subpopulation is intrinsically as sensitive to IFN action as VSV, whereas others are up to 100-fold more resistant, albeit on a transient, nongenetic basis.[22] NDV is relatively resistant to IFN action, even up to doses of 2000 U/ml (see Fig. 5), whereas ARV is extremely resistant, with over 80% of the population resisting high doses of IFN (2000 U/ml for 24 h) (see Fig. 3).

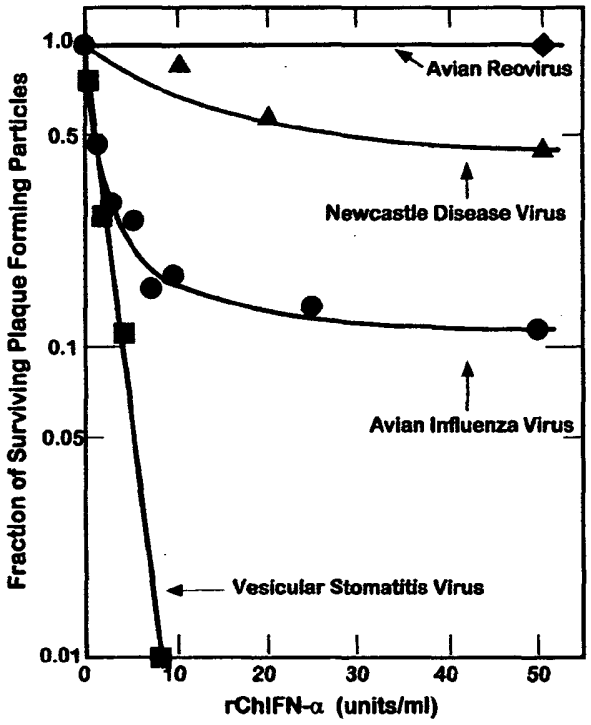

FIG. 1. Plaque-reduction assays of viruses with different sensitivities to ChIFN. Monolayers of primary CEC were exposed to different concentrations of rChIFN-α for 24 h at 37.5°C for development of the antiviral state, challenged with virus, and incubated at 37.5°C for plaque development. Monolayers were stained vitally with neutral red, and the number of surviving plaques was counted as described previously.[20]

Combined sequential action of IFN and dsRNA overcomes ARV resistance to IFN

The basic observation on which this report is based is illustrated in Figure 2. Plaque assays of ARV, of which these are typical, were run in quadruple and found to agree within ± 15% of the mean value. They demonstrate that the action of IFN (2000 U/ml for 24 h) by itself or dsRNA in the form of poly(rI):poly(rC) (1000 ng/ml for 1 h) by itself had a modest effect on plaque formation by ARV (Fig. 2A, untreated cells]; Fig. 2B, IFN only; Fig. 2C, poly(rI):poly(rC) only). In marked contrast, Figure 2D shows that when CEC were treated first with IFN for 24 h and then with poly(rI):poly(rC) for 1 h before plaque assay, there was a marked reduction in the resistance of ARV to these two biologic response modifiers acting in concert; that is, a high-level antiviral state had developed. The cytotoxicity/apoptosis induced by dsRNA in many kinds of IFN-treated cells[28] or that which is intrinsic to dsRNA itself in some cells[29] was not observed in CEC, as evidenced by the distinct plaque areas in monolayers stained vitally with neutral red (compare Fig. 2C and 2D with 2A and 2B).

Abrogation of resistance of ARV to IFN action is dsRNA dose dependent

Figure 3 shows data typical of four independent experiments. The fraction of surviving ARV plaques in cells treated only with rChIFN-α (2000 U/ml for 24 h) was plotted relative to untreated virus-infected cells. Data points for doses of IFN < 2000 U/ml differed by ± 15% from virus controls and are not shown. Extrapolation of the ARV plaque-forming particles (PFP) survival curve in cells treated only with IFN showed that 4200 U/ml IFN would be required to reduce plaque formation by 50%. The titer of the IFN is determined with VSV as the challenge virus and is normalized against an IFN standard in each experiment to assess the sensitivity of a given batch of primary CEC to IFN action.[30] This means that ARV is about 4200-fold more resistant to ChIFN than is VSV.

Figure 3 also illustrates the survival of ARV PFP in CEC monolayers exposed only to the dsRNA poly(rI):poly(rC) (1000 ng/ml for 1 h). Data points for concentrations of poly(rI):poly(rC) < 1000 ng/ml were within 15% of control values and are not shown. Although this treatment results in the coinduction of a small amount of an acid-labile IFN along with the

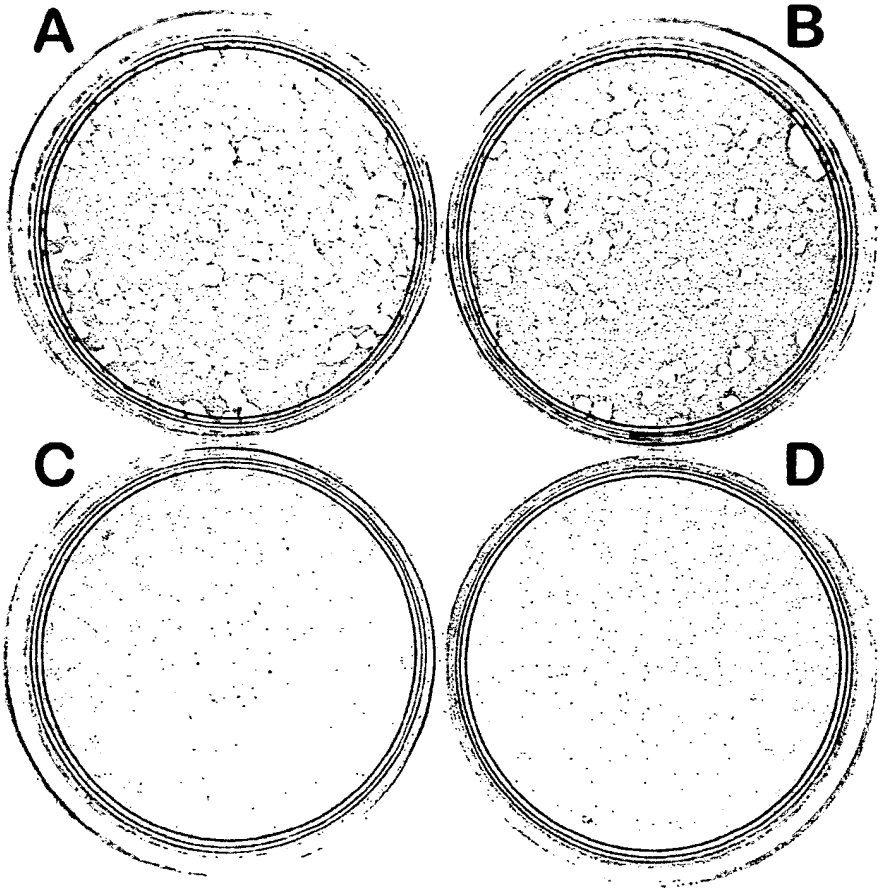

FIG. 2. Plaque formation by ARV-S1133[17] on monolayers of primary CEC.[20] (**A**) Untreated. (**B**) IFN only at 2000 U/ml for 24 h. (C) Poly(rI):poly(rC) only at 1000 ng/ml for 1 h. (**D**) IFN at 2000 U/ml for 24 h, followed by poly(rI):poly(rC) at 1000 ng/ml for 1 h. IFN and poly(rI):poly(rC) treatments were at 37.5°C for development of an antiviral state and plaque formation. Neutral red vital staining was used to better visualize the plaques. Actual size.

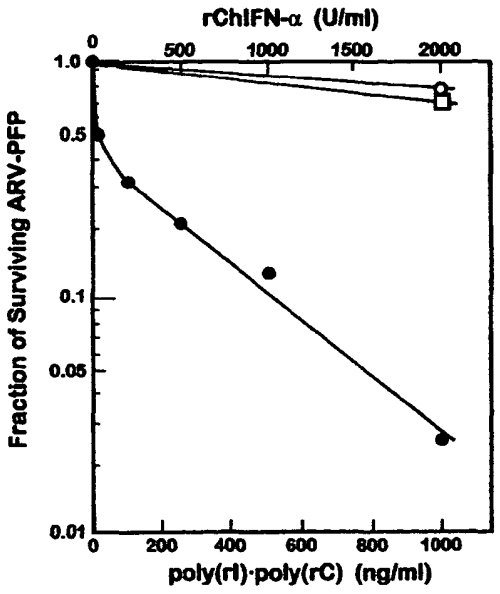

FIG. 3. Abrogation of the anti-IFN action of ARV by exogenously added dsRNA in the form of poly(rI):poly(rC). Monolayers of primary chicken cells were treated for 24 h only with 2000 U/ml rChIFN-α (open circle) or for 1 h only with 1000 ng/ml poly(rI):poly(rC) (open square). A third set of cells was first exposed for 24 h to 2000 U/ml IFN and washed and then received a 1-h exposure to different concentrations of poly(rI):poly(rC)(solid circles). All plates were challenged with the same number of ARV PFP, overlaid with a medium-agarose mixture, and 3 days later stained with neutral red. The number of plaques was averaged from triplicate plates. The fraction of surviving ARV PFP is plotted as a function of poly(rI):poly(rC) concentration.

usual acid-stable IFN[31] and a dsRNase,[26] there is only a modest decline in the surviving fraction of ARV PFP. Treatment with poly(rI):poly(rC), carried out to 5000 ng/ml for 1 h in two experiments, was not toxic to CEC and revealed $PR_{50} \cong 2500$ ng/ml of the polyribonucleotide Treatment of CEC with DEAE-dextran (10 μg/ml) by itself or with poly(rI):poly(rC) degraded by dsRNase[26] had no effect on the plaquing efficiency of ARV (data not shown). The use of poly(rI):poly(rC) stabilized with polysine and carboxymethylcellulose (poly-ICLC)[26] gave results comparable to the poly(rI):poly(rC)-DEAE-dextran complex when used in equimolar amounts of dsRNA (data not shown).

The remaining curve in Figure 3 shows that in contrast to treatment with IFN or poly(rI):poly(rC) alone, when CEC were first exposed to a constant amount of IFN for 24 h (2000 U/ml) and subsequently to various concentrations of poly(rI):poly(rC) for 1 h, there was a dsRNA dose-dependent reduction in the fraction of surviving PFP. This defined a biphasic survival curve. About one half of the virus population was highly sensitive to the exogenous addition of dsRNA to the IFN-treated cells and revealed a value of $PR_{50} \cong 25$ ng/ml poly(rI):poly(rC). This represents an approximately 100-fold increase in the effectiveness of the dsRNA compared with its use as a stand-alone reagent. The remaining half of the ARV population was about 10-fold less sensitive to the presence of poly(rI):poly(rC) in the IFN-treated cells. Nonetheless, even this decrease in sensitivity resulted in a 40-fold reduction in ARV plaque count at 1000 ng/ml poly(rI):poly(rC) when compared with either treatment alone.

ARV resistance to IFN is not overcome if dsRNA is added before the IFN-mediated latent antiviral state is established

Figure 4 shows that the resistance of ARV to the action of IFN is not altered significantly if poly(rI):poly(rC) is added 1 h before rather than after the 24-h treatment with IFN. Controls consisting of poly(rI):poly(rC) by itself added for 1 h temporally before or after a 24-h mock treatment with IFN are similar.

Resistance of NDV to IFN action is reduced by dsRNA in a dose-dependent manner

NDV, a paramyxovirus, was tested for its sensitivity to the combined sequential treatment of IFN and dsRNA because it is relatively refractory to the action of IFN (Fig. 1), and we could find no reports that it produced a dsRNA-binding protein or contained a putative dsRNA-binding sequence. Figure 5 shows that when used separately, 2000 U/ml IFN or 1000 ng/ml

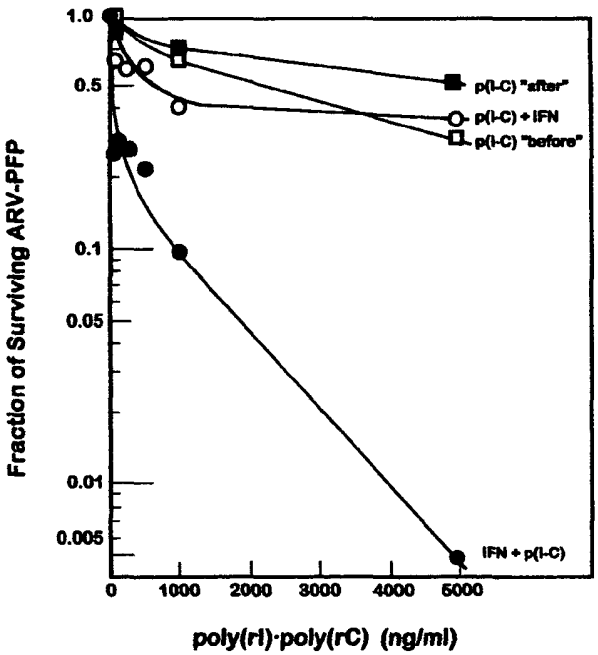

FIG. 4. Poly(rI):poly(rC) added before the IFN-mediated antiviral state is established does not overcome the resistance of ARV to IFN. Experimental conditions are the same as described in the legend to Figure 3 except that the poly(rI):poly (rC) was added 1 h before rather than after the IFN-mediated antiviral state was established. Control cultures of CEC were treated with different concentrations of poly (rI):poly(rC) before (open squares) or after (solid squares) mock addition of IFN for 24 h. Other sets of cells were exposed for 1 h to poly(rI):poly(rC) before (open circles) or after (solid circles) actual treatment for 24 h with 2000 U/ml rChIFN-α.

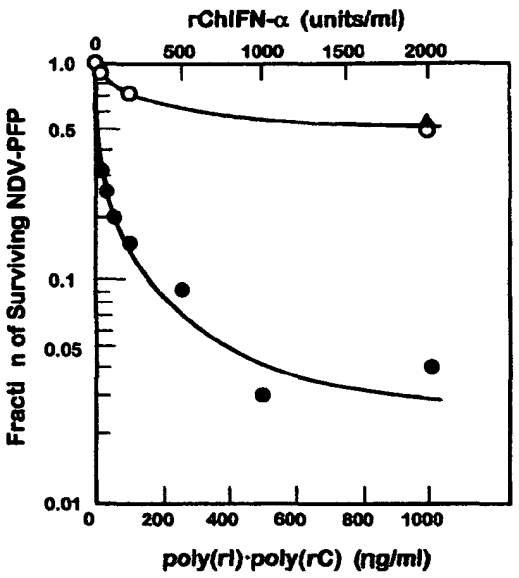

FIG. 5. The intrinsic resistance of NDV to the antiviral action of IFN is reduced significantly by the exogenous addition of poly(rI):poly(rC). Monolayers of CEC were treated with different concentrations of only rChIFN-α for 24 h (solid triangle), only poly(rI):poly(rC) for 1 h (open circles), or the combination of these two biologic response modifiers in the sequence of IFN followed by poly(rI):poly(rC) (solid circles). Conditions were otherwise as described in the legend of Figure 3.

poly(rI):poly(rC) each reduced the plaquing efficiency of NDV by about 50%. The combined treatment in which the dose of IFN was kept constant at 2000 U/ml for 24 h and the concentration of poly(rI):poly(rC) was varied for the 1-h treatment that followed showed that about 60% of the NDV population was quite sensitive, displaying a value for $PR_{50} \cong 20$ ng/ml poly(rI):poly(rC), a 50-fold increase in sensitivity. The remaining subpopulation of NDV became disproportionately less sensitive to increasing concentrations of poly(rI):poly(rC). Nonetheless, there was a 15-fold increase in sensitivity observed at 1000 ng/ml dsRNA in the IFN-treated cells. Again, the combined treatment was more efficacious than either reagent acting alone against NDV.

DISCUSSION

These data extend earlier reports that ARV is extremely resistant to the action of IFN and show that dsRNA in the form of high molecular weight poly(rI):poly(rC) complexed with DEAE-dextran, or as polyICLC, was no better alone than was IFN in developing an antiviral state against ARV. However, we demonstrate that combined treatment with these two biologic modifiers presented to cells in the sequence IFN first followed by dsRNA results in a dsRNA dose-dependent abrogation of IFN resistance that renders ARV up to 100 times more sensitive to IFN action than to either IFN or dsRNA acting alone. Comparable results were obtained with a paramyxovirus, NDV, demonstrating that this combined IFN and dsRNA treatment was active against viruses from two very different families. In the case of ARV, we interpret these results in light of the avid dsRNA-binding capacity of ARV σA protein demonstrated *in vitro*[7] and by its mammalian equivalent, σ3, as shown when substituted for E3L, the dsRNA-binding protein expressed in vaccinia virus-infected cells.[16] We conclude that under these conditions, insufficient amounts of free dsRNA are available to reach the threshold required to activate latent dsRNA-dependent antiviral mechanisms and that the exogenously added dsRNA exceeds the binding capacity of the σA protein extant in ARV-infected cells, and that dsRNA no longer is rate limiting.

To be effective, the dsRNA has to be added after establishment of the IFN-mediated latent antiviral state. Exposure of cells to dsRNA prior to development of the latent antiviral state was ineffective. We postulate that the dsRNA has a short half-life in the cell and, hence, must be present when the dsRNA-dependent reactants of the IFN-mediated latent antiviral state are abundant, that is, after their induction. In the case of viruses whose gene products lack dsRNA-binding capacity, as may be the case with NDV, we postulate that dsRNA induces some unique dsRNA-stimulated gene(s)[32–38] whose product acts synergistically with some IFN-stimulated gene product(s)[39] to enhance the antiviral effect. Synergy is invoked, since as we have shown, the action of dsRNA of itself does not overcome the intrinsic resistance of ARV or NDV to IFN action. Synergy of action between mammalian types I and II IFN is well established[40] and recently has been reported to occur in chicken cells.[24] This proposed mode of action and one based on overcoming rate-limiting concentrations of dsRNA are not mutually exclusive and may act in concert. In this context, prior treatment of human endothelial cells with HuIFN-α enhanced the activation by poly(rI):poly(rC) of several cytokines involved in the cellular response to viral infection.[38]

In some cells, apoptosis may contribute to the antiviral effects of IFN,[41] with subsequent dsRNA treatment known to exacerbate the effect.[28] However, not all cells display apoptosis in response to the dual exposure to IFN and dsRNA. Of five lines of mouse L cell lines obtained from as many investigators, only one demonstrated measurable cell killing by the loss of colony-forming capacity (unpublished observations). In this context, primary CEC appear unaffected by the dual exposure to IFN and dsRNA, as evidenced by the uptake of neutral red under vital staining conditions (Fig. 2). Furthermore, the formation of normal-size plaques of virus as survivors on the doubly treated cells implies that cellular macromolecular synthesis is not compromised and replication of the virus is unaffected. The plaque areas involve about 5,000–10,000 cells at the time of staining, from which we infer that virtually all cell types in the heterogeneous mixture of primary embryonic chicken cells respond to viral replication with a cytopathic effect (Fig. 2). The absence of apoptosis in CEC treated with IFN and then dsRNA, as assessed by vital staining with neutral red and viral replication capacity, may reflect a short $T^1/_2$ of the dsRNA because of a potent dsRNase induced in CEC when exposed to dsRNA or viral inducers of IFN.[26]

The biphasic or multiphasic virus survival curves might reflect the myriad cell types that constitute a population of primary CEC[30] or the quasispecies nature of these RNA viruses and their response to the IFN system.[23] We favor the latter possibility, as similar triphasic survival curves for resistance to IFN action were generated by avian influenza virus in both the

heterogeneous cell population of primary CEC and the relatively uniform LSCC line of quail cells.[22]

Viruses from other families have evolved defense strategies to abrogate IFN action mechanisms based on the sequestration of dsRNA, with the PKR system targeted in particular,[2–5] for example, influenza virus NS1[22,42,43] and vaccinia virus *E3L*,[16,44] and, hence, are potential candidates for the combined IFN/dsRNA treatment described herein. Interestingly, in *E3L*-deficient vaccinia virus, expression of RNase III restored the IFN-resistant phenotype.[45] From our view, the intracellular action of dsRNase, along with its dsRNA binding capacity, represents another means of reducing the dsRNA levels below the threshold required for activation of the IFN-mediated antiviral state.

Another anti-IFN mechanism involves the action of viral-produced small helical RNAs that bind to PKR without activating it but may prevent activation by full-size dsRNA, for example, HIV TAR, Epstein-Barr virus EBER-1, and adenovirus VAI (reviewed in ref. 46). Exogenously added dsRNA might outcompete these RNA analogs. Possible synergy between IFN-stimulated gene products and those induced exclusively by dsRNA may extend the list of viruses potentially sensitive to the combined sequential application of IFN and dsRNA described herein. Thus, it may be found useful in overcoming the anti-IFN activity of viruses of clinical interest and even find relevance in other clinical conditions where IFN by itself is marginally, if at all, effective.

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